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SECTION 38(1) 1977 ACT) APPLICATION FILED 22.9.98
23 ST MACHAR DRIVE
ABERDEEN AB24 3RY

Patents ADP number *(if you know it)*

If the applicant is a corporate body, give the country/state of its incorporation
INCORP. IN SCOTLAND 6622203006

4. Title of the invention
RIBOSOME DISPLAY

5. Name of your agent *(if you have one)*

"Address for service" in the United Kingdom to which all correspondence should be sent *(including the postcode)*
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11. I/We request the grant of a patent on the basis of this application.

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RIBOSOME DISPLAY

The present invention relates to methods and compositions for the generation and screening of combinatorial libraries of displayed polypeptides encoded by natural or artificial DNA sequences which are expressed on polysomes following in vitro transcription and translation. In particular, the invention relates to the efficient expression of full-length polypeptides on polysomes attached to the encoding mRNA sequence and the subsequent determination of that sequence.

The ribosome represents a collection of proteins whose co-ordinated activities accomplish the act of translation. The basic form of the ribosome is conserved, though there are appreciable differences in the size and proportions of RNA and proteins in the ribosomes of prokaryotes and eukaryotes, and in organelles. All ribosomes consist of two main subunits, in bacteria a 50S and 30S, in eukaryotes a 60s and 40S subunit. Protein synthesis starts when the ribosome attaches to mRNA either at the 5' cap in eukaryotes or adjacent to the translational initiation codon in prokaryotes (usually AUG) and continues by the successive loading, directed by the mRNA sequence, of amino acids onto a peptidyl-tRNA molecule carrying the nascent polypeptide chain. Translation terminates usually at a stop codon where the ribosome disassociates from the mRNA. In the absence of a stop codon, the ribosome is thought to progress to the end of the mRNA molecule before disassociation occurs with the assistance of release factors. Translation termination therefore disconnects the protein from the mRNA molecule encoding it. If the ribosome is arrested during protein synthesis, then the protein and mRNA will remain connected whilst complexed with the ribosome.

Methods of selecting proteins as the carrier of a particular phenotype and subsequently determining the corresponding genotype have relied mainly on living cells to provide a link between genes and proteins commonly using bacteriophage, viruses and bacterial cells displaying the desired protein. For a diverse collection of proteins represented by a DNA library, the use of live cells has several disadvantages. For example, the protein diversity can be reduced by the requirement for transformation or infection of bacterial or eukaryotic cells which is limited by the low efficiency of DNA or infectious particle uptake. Furthermore, the biological production of diverse proteins is subject to the particular environment of the living cell which can select against certain proteins and can lead to the variations in protein folding which can also select against certain proteins. Finally, if diversification of individual proteins during successive selection rounds is required, genetic mutation techniques are difficult to apply to *in vivo* systems due to the need to switch between DNA and live cells for the diversification and screening.

In vitro methods for the selection of a polypeptide potentially offer advantages over *in vivo* methods by eliminating the need for uptake of genes into cells and by controlling the environment for mRNA and protein production. *In vitro* transcription and translation reactions have been used for many years as a means of generating polypeptides directly from DNA and it has been shown that specific mRNAs can be enriched by the immunoprecipitation of polysomes (e.g. Payvar, F. and Schimke, R. T., Eur. J. Biochem., vol 101 (1979) p1844-1848) using antibodies to select the specific polypeptides. This so-called "ribosome display" technique has more recently

been adapted for the selection of peptides (Mattheakis. L. *et al*, PNAS **91**: 9022, 1994 and PCT95/11922) and proteins (Kawasaki, G., PCT91/05058 and Hanes & Pluckthun, PNAS. **94** [10]:4937, 1997).

The method described by Mattheakis. L. *et al* (PNAS **91**: 9022, 1994) uses ribosome display systems for identification of ligands from peptide libraries. The method uses chloramphenicol for ribosome translation arrest which induces stalling in ribosome translation by binding the 50S subunit of the prokaryotic ribosome complex. A disadvantage of this method is that it will cause translation arrest regardless of the length of the nascent peptide and thus most polypeptide molecules will be incomplete. For efficient screening of polypeptides from a DNA library following transcription/translation, it is clearly desirable to maximise the yield of full-length polypeptides associated with mRNA.

The methods described by Mattheakis and Dower in PCT95/11922 include additional measures to stall ribosomes principally throught the use of "Tethered Nascent Peptides" which are peptide portions of a fusion polypeptide molecule, adjacent to the polypeptide portion for screening, which interact or bind with the encoding polynucleotide in order to maximise the yield of polypeptides associated with mRNA. As with Mattheakis. L. *et al* (PNAS, ibid), this method would be expected to cause premature translational arrest and would also be expected to distort the proper folding of some proteins for screening. Thus, a range of sizes of polypeptide molecules would be expected by the methods described by Mattheakis and Dower which would reduce the probability of isolating specific polypeptides encoded by the DNA library.

In the method of Hanes & Plucktun (ibid), there is provided a method to optimise the yield of correctly folded protein and its encoding mRNA while both are still attached to the ribosome. The method includes stalling the translation process by increasing magnesium acetate concentration and correct folding of proteins by the manipulation of the physical reaction conditions. However, the only measure to increase the yield of full-length protein is to eliminate stop codons from the mRNA by manipulating the corresponding genes and adding a 3' spacer region in order to tether the folded protein on the ribosome. Whilst this method should increase the proportion of full-length polypeptides when compared to earlier methods, the method provides no measures to prevent run-off translation from the end of the mRNA and the stalling strategies provided suffer from the same limitations as with Mattheakis and Dower (ibid) whereby premature translational arrest would be expected.

In addition to the limitations described ofr each of the methods above, none of the methods provide a means to block new translational starts late in the translation reaction prior to testing of translated polypeptides. Thus, a range of sizes of polypeptide molecules would be expected by these methods which would reduce the probability of isolating specific polypeptides encoded by the DNA library. There is therefore a need for new methods for ribosome display which maximise the yield of full-length polypeptides whilst minimising the presence of nascent incomplete polypeptide chains in order to efficiently screen a DNA library for specific polypeitides.

The present invention provides a new *in vitro* method of ribosome display which maximises the expression of full-length polypeptides from a ribosome by selective arrest of mRNA translation at the 3' end of the mRNA such that the ribosome complex it is still linked to the full-length polypeptide. The invention is based on the discovery that proteins bound to specific sites in mRNA molecules will block the further translation of the mRNA allowing the stalling of a mRNA-ribosome complex with associated polypeptide. The invention also includes optional measures to prevent new translational starts just prior to polypeptide screening.

The method of the present invention involves firstly the creation of a library of DNA molecules, commonly within a plasmid vector, such DNA molecules encoding various polypeptides whereby the DNA encoding these polypeptides is transcribed into mRNA molecules. Commonly, the cloning vector for the DNA molecules includes an upstream promotor such as a promotor for T7 RNA polymerase. Thus, the pooled library of DNA molecules is transcribed, for example by the addition of T7 RNA polymerase and ribonucleotides, to produce a library of mRNA molecules. Thereafter, the library of RNA molecules is translated using a ribosome preparation such as an E.coli S-30 fraction (Chen H Z and Zubay G, Methods in Enzymology, vol 101 (1983) p674-690). Thereafter, the ribosome complexes, comprising linked polypeptide, ribosome and mRNA, are typically screened for binding to a ligand immobilised onto a solid phase and mRNA is released from the resultant immobilised complexes for reverse transcription and amplification by PCR in order to enrich for DNA molecules encoding polypeptides which bind to the target ligand. These DNA molecules can either be used directly for transcription or can be cloned in order to determine the sequences of DNA encoding polypeptides which bind to the target ligand.

For the purpose of this invention, the mRNA encoded by the DNA molecules will be considered to include 3 segments comprising, from 5' to 3', a variable segment, a spacer segment and a termination segment with an optional anti-initiation segment 5' to the variable segment. The polypeptide shall mean the protein or peptide sequence translated from the variable segment of the mRNA to protein via a ribosome complex. The variable segment shall mean mRNA sequences encoding the full-length polypeptide. The spacer segment shall mean mRNA sequences encoding a protein segments contiguous with the variable segments which allow the completed proteins to completely emerge from the ribosome and adopt optimal three dimensional structures whilst still attached to the encoding mRNA through the ribosome complex. The terminating segment shall mean mRNA sequences to which the binding moiety attaches either directly or indirectly or, alternatively, mRNA sequences encoding a polypeptide binding moiety, contiguous with or upstream from the spacer segment to which specific proteisn attach in order to block translation.. The binding moiety shall mean any molecule that can bind either to the mRNA on the ribosomal complex or any molecule that can bind to the translated polypeptide on the ribosomal complex resulting in arrest of translation. The binding moiety will usually bind directly to mRNA in a sequence specific manner or may be bound indirectly to the mRNA, for example after annealing of a synthetic DNA or RNA molecule to the mRNA and addition of the binding moiety via a ligand on these molecules. The optional anti-initiation segment will be adjacent to the translational initiation codon and will

provide sequences for attachment usually of a different binding moiety. The anti-initiation segment will prevent new translational initiations just prior to screening of the translated polypeptides. The target ligand will be the molecule against which the library is screened for binding of specific proteins and subsequent recovery of associated mRNA.

The variable segment of the mRNA molecules comprises either full-length known polypeptide types such as single-chain antibodies (comprising immunoglobulin variable regions derived from heavy and light chains and linked together to give a functional binding domain), or random or semi-random sequences. Chimaeric sequences created by random/semi-random association of known polypeptide types or regions may also be used. For known polypeptide types such as single-chain antibodies, specific segments of the genes may be randomised such as the CDRs in single-chain antibodies. Large collections of DNA molecules encoding known, random or semi-random sequences will be firstly produced and then cloned into the transcription vector. This transcription vector will provide other segments for inclusion in the subsequent mRNA molecules as detailed below. The vector will also usually provide a translation initiation codon and ribosome binding site for the mRNA. For longer polypeptide molecules encoded by the DNA, it will be necessary to reduce or eliminate the presence of stop codons in the mRNA which would terminate translation. It is a requirment of the invention herein that such stop codons, including stop codons natural to specific proteins, are eliminated in order to yield full-length polypeptides. For DNA encoding known proteins such as single-chain antibodies, this will simply require elimination of the usual stop codon or, alternatively, the use of nonsense suppressing tRNAs in the translation reaction in order to insert specific amino acids at the position of the stop codons. For random or semi-random DNA produced by chemical synthesis, the frequency of stop codons occurring in any particular sequence can be reduced by manipulation of DNA base composition in the synthesis reaction and nonsense suppressing tRNAs could also be used in the translation reaction.

The spacer segment of the mRNA molecules provides a polypeptide region downstream of the variable segment which spans the channel of the ribosome in order to permit the full-length polypeptide to fully emerge from the ribosome to allow for proper protein folding and unhindered access to the target ligand. Usually this segment will encode a region of over 50 amino acids and will encode a region which is unlikely to interfere with folding of the full-length polypeptide such as a complete domain from another protein or a glycine/alanine-rich linker such as $(Gly4Ser)_{10}$. For some translated proteins, the spacer segment may comprise a contiguous portion from the protein itself where this portion is not required for correct folding of the variable segment or for binding to the target ligand.

The terminating segment provides a downstream mRNA sequence for direct or indirect attachment of a binding moiety which is designed to prevent translational termination following translation of the complete variable segment. One example of a binding moeity which binds to a terminating segment in mRNA is the Iron Regulatory Protein (IRP). The terminating segment would provide a stem-loop structure which is stabilised by the addition of the IRP in conditions of low iron concentration, thus resulting in a steric block to the ribosome translation. Following

this selection of the nascent peptide can occur. The use of IRP to arrest the ribosome introduces a reversible block such that addition of iron may allow translation to resume and thus terminate. This would facilitate the release of the mRNA after selection of the polypeptide for subsequent transcription, sequencing or cDNA cloning. Further examples of mRNA binding moieties binding to sequences in the terminating segment include the HIV protein tat, which binds to a RNA stem-loop termed TAR (Dingwall et al., PNAS, vol 86 (1989) p6925-6929), La antigen which binds to a RNP motif (Chan E K L and Tan E M, Mol. Cell. Biol. vol 7 (1987) p2588-2591) and other proteins from RNA viruses which bind to specific RNA sequences either in single- or double-stranded RNA, the latter which can be created in mRNA via hairpin loops. The terminating segment alternatively could encode a site for attachment of a binding moiety to the polypeptide in order to prevent translational termination. Optionally, the terminating segment will include one or more further regions of mRNA which increase the stability of mRNA against exonucleases such as the lpp (E.coli lipoprotein) and phage T3 terminators.

The optional anti-initiation segment adjacent is designed to prevent translational initiations late in the translation reaction which might not result in full-length polypeptides and might therefore provide polypeptides with incomplete folding which might provide non-specific binding to target ligands. The anti-initiation segment might encode, for example, a secretory leader sequence in the translated polypeptide to which can be bound the signal recognition protein (SRP). Once SRP has bound to the polypeptide, it causes the arrest of further translation by cross-linking of the polypeptide and the mRNA. Translational arrest may also be achieved by using a combination of the SRP54 and SRP9/14 subunits of SRP (Siegel & Walter, Cell Biol., vol 100 (1985) 1913-1921) which bind to the nascent peptide and mRNA respectively. Another example of anti-initiation sequences is provided by certain eukaryotic transcription leaders such as that of the human cytomegalovirus gp48 gene which contains an upstream 22 codon which represses translation of the downstream cistron (Cao J and Geballe A P, Mol. Cell. Biol. vol 16 (1996) p7109-7114). Such leaders are thought to encode peptides which block ribosome progression to the downstream cistron. Further examples of anti-initiation sequences are sequences recognised by binding moieties such as for the terminating segment as above, these including sequences bound by IRP, tat, La antigen and other viral proteins.

The invention therefore provides compositions of DNA libraries encoding mRNA molecules with variable, spacer and terminating segments with an optional anti-initiation segment. Preferably, the invention provides a DNA vector encoding the spacer and terminating segments with the optional anti-initiation segment. Commonly, the DNA vector will also provide the translation initiation codon and, for translations using prokaryotic ribosomes, a ribosome binding site including the Shine-Dalgarno sequence. For eukaryotic translation systems, the Kozak translation initiation sequence with consensus GCCGCCACCATGG may also be included and, upstream from the translation initiation site, it may be desirable to include other known sequences to enhance translation such as enhacers or activator sequences including untranslated leader sequences from certain viruses such as tobacco mosaic virus. Commonly, the DNA vector will also provide a strong transcriptional promotor which will be used in conjunction with a RNA polymerase to produce a library of mRNA molecules corresponding to the DNA library. Such promotors will include

those for T7 RNA polymerase, T3 RNA polymerase and SP6 RNA polymerase and, additionally, a promotor for the RNA dependant polymerase, Qb replicase, may also be encoded by the DNA. The DNA vector will also provide a strong transcriptional terminator, for example the terminator of E coli lipoprotein or the early terminator of phage T3. In the method of the invention, DNA fragments containing the variable segments are cloned into the DNA vector whereby the DNA fragments have a minimum of or no stop codons. For libraries encoding known polypeptide types such as single-chain antibodies, the DNA fragments will be cloned unidirectionally using appropriate restriction sites. It will be apparent to those skilled in the art that a variety of replicable DNA vectors could be used in the method of the present invention including plasmid, bacteriophage, phagmid and viral vectors. It will also be clear that DNA amplification by methods such as PCR could be used as an alternative to replication of DNA in living cells. It will also be clear that the variable segments to create the DNA library could be provided from a number of sources including a vector library of DNA fragments, synthetic DNA or amplified DNA. In addition, the variable segments could be provided as a result of mutagenesis reactions using a fixed template, for example using error-prone PCR. It will also be clear that the variable segments could be composed of DNA directly from the genome of a living organism or from cDNA copies of mRNAs of that organism. For example, where the DNA library comprises single-chain antibody fragments, these fragments could be derived from mRNA encoding immunoglobulin variable regions and expressed by B cells within an organism. Alternatively, the fragments could be derived from genomic variable regions. In such manner, the invention will provide for the creation of single-chain antibody libraries from specific organisms such as man.

The invention also provides compositions of libraries of mRNA molecules with variable, spacer and terminating segments with an optional anti-initiation segment. Preferably, the invention provides a library of mRNA molecules encoding the spacer and terminating segments with the optional anti-initiation sequence. The mRNA molecules will each include a translation initiation codon and, for translations using prokaryotic ribosomes, a ribosome binding site including the Shine-Dalgarno sequence. For translation using eukaryotic ribosomes, the mRNA may synthesised with a 5' capping nucleotide or this may be introduced enzymatically into the pre-synthesised mRNA. Upstream from the translation initiation site, the consensus Kozak translation initiation sequence may also be included and other known sequences to enhance translation such as enhancers or activator sequences including untranslated leader sequences from certain viruses. Prior to or during the process of translation, one or more binding moieties will become associated with the terminating segment of the mRNA molecules thus stalling translation. Also, during the process of translation, one or more binding moieties may become associated with the optional anti-initiation segment within or encoded by the mRNA molecules thus preventing new translational initiations.

The invention also provides compositions of libraries of translated polypeptide molecules with variable and spacer segments with an optional anti-initiation leader sequence. These protein molecules will provide full-length variable segments as part of a longer polypeptide chain which is stalled within the ribosomes through the interaction of binding moieties with the mRNA. At the 5' end of the polypeptide chain, there may also be a site for association of a binding moiety, such as SRP,

which interacts with the ribosome thus preventing additional translation on the mRNA molecule.

Within the method of the present invention, it will be apparent to those skilled in the art that various measures could be used to optimise the stability of mRNA molecules especially from degradation by RNAses. For example, various inhibitors of RNAses such as Rnasin and vanadyl ribonucleoside complexes could be used in the transcription reactions. Alternatively or additionally, various structures could be included in the mRNA molecules including 3' stem-loops such as those commonly provided by transcriptional terminators. It will also be apparent to those skilled in the art that transcription and translation reactions might either be performed separately or, especially with prokaryotic systems, combined into a coupled *in vitro* transcription/translation reaction. Preferably, the transcription and translation reactions will be performed separately in order to optimise the production of mRNA and polypeptides which will require different optimal reagents.

Within the method of the present invention, it will be apparent to those skilled in the art that various measures could be used to optimise the folding and stability of protein molecules especially from degradation by proteases. For correct protein folding, optimal oxidative protein folding conditions would be used in translation reactions with particular measures to optimise disulphide bond formation through, for example, use of molecular chaperones such as protein disulphide isomerase. For stability, the peptide tagging system of E.coli could be disabled by inhibition of *ssrA* RNA using methods such as that of Hanes and Pluckthun (ibid). Once the final polypeptide/ribosome/mRNA complexes are formed, it may be beneficial to stabilise these complexes using standard translational inhibitors such as chloramphenicol. Additional chemical or photochemical cross-linking of the polypeptide chain and/or mRNA to the ribosome may also be beneficial.

Once polypeptide/ribosome/mRNA complexes are formed, the mixture is then screened for binding to the target ligand in such a manner that successfully binding complexes can subsequently be recovered. This could be achieved by virtue of binding to a target ligand immobilised onto a solid phase such as a plastic or glass surface or the surface of a latex or magnetic bead. This could also be achieved by virtue of the binding of the target ligand effecting another reaction or series of reactions which subsequently provides the basis for separation of the complex, for example where binding to the target ligand on the surface of a cell causes a change in that cell such as the appearance of a surface antigen whereby the cell can be separated from other cells in the population along with the bound polypeptide/ribosome/mRNA complex. As an alternative, the polypeptide could be screened for a particular enzymic activity whereby the target ligand is, for example, an enzyme substrate which irreversibly binds to the enzyme or where the substrate is converted by the enzyme to a product which can combine with the polypeptide/ribosome/mRNA complex or can effect some other change such that the polypeptide/ribosome/mRNA complex including the enzymic activity can be separated from the total mixture of complexes.

Once polypeptide/ribosome/mRNA complexes are separated by virtue of binding to a target ligand, the complexes can either be purified preparatively by standard methods or the RNA simply released using, for example, EDTA or simple heating prior to

reverse transcription into cDNA and, preferably, amplification by methods such as PCR. The amplified DNA can then either be transcribed and translated again in order to further divide the library or can be cloned to produce a sublibrary. For molecular evolution strategies where polypeptides which bind to the target ligand are evolved by mutation, mutagenesis can either be effected at the stage of cDNA amplification using, for example, error-prone PCR or can be effected by subjecting the cloned sublibrary to mutagenesis, for example using mixed oligonucleotides to mutagenise specific regions of the polypeptide-encoding DNA segment. If required, the initial DNA library for transcription/translation or a sublibrary following screening can be subdivided into pools or individual clones for screening in order to reduce the complexity of the mixture of polypeptides subjected to screening. After finally identifying one or more polypeptides deriving from the DNA library, DNA encoding the specific polypeptides of interest can then be subcloned into expression vectors in order to produce high levels of the desired protein without, as required, the spacer segment or any other flanking segments.

It will be apparent to those skilled in the art that the present invention will have a variety of applications including the isolation of variants of known polypeptide types such as single-chain antibodies or totally novel proteins with biochemical or biological activities superior to those of existing proteins. Chimaeric proteins created as hybrids of regions from known polypeptides and regions of novel polypeptide sequence may also be produced. For multichain polypeptide types such as antibodies, functional or novel combinations of heavy and light chain variable regions may also be produced.

The following examples are provided to illustrate the invention and should not be considered as limiting the scope of the invention.

Example 1

The starting point was two single-chain antibody genes (scFv) specific for the epidermal growth factor receptor (EGFR) and for the prostate membrane surface antigen (PMSA) each cloned into an E.coli expression vector, pPMhis. The scFv fragments were recloned into a pGEM T7/SP6 plasmid (Promega, Southampton, UK) providing an upstream promotor for T7 RNA polymerase using long synthetic oligonucleotides and PCR to provide an upstream bacterial ribosome binding site, a downstream spacer segment derived from the M13 phage gene III and a 3' transcriptional termination region from the E.coli lpp terminator. For both scFv's, the translational stop codon was removed by the PCR reaction. The resultant plasmids were called pT7340A (for EGFR) and pT7591A (for PMSA).

As variants of the basic plasmids above, an upstream anti-initiation segment and a downstream terminating segment and were provided by SOE PCR to insert, respectively, a secretory leader sequence between the ATG initiation codon and the scFv genes, and a terminal sequence TAR sequence between the spacer segment and the transcriptional termination sequence. The resultant plasmids were called pT7340B (for EGFR) and pT7591B (for PMSA).

In vitro transcription of the pT7 plasmids was performed using a RiboMAX™ kit (Promega, Southampton, UK) according to the manufacturer's instructions. The resultant mRNA was purified according to the manufacturer's protocol.

In vitro translation was performed in an E.coli S-30 system as described by Chen and Zubay (ibid) modified as described by Hanes and Pluckthun (ibid) and supplemented with HIV Tat 37-72 peptide. After 10 minutes of translation, a preparation of SRP (produced by the method of Romisch K, Webb J, Herz J, Prehn S, Frank R, Brenner S and Walter P, Nature vol 340 (1989) p478-482) was added and the translation was continued for a further 10 minutes. The translation was stopped and the mixture centrifuged as described by Hanes and Pluckthun (ibid). The translation reactions were then incubated for 1 hour at room temperature on microtitre plates with either EGFR or PMSA coated into the plates. Washing and dissociation of retained ribosome complexes, isolation of mRNA, reverse transcription-PCR and repeated transcription-translation were as described by Hanes and Pluckthun (ibid). After 5 rounds of ribosome display, the PCR products were cloned into pUC18 for sequence determination and determination of the representation of the original scFv's in the selected population of genes. For this determination, the inserts in at least 50 pUC18 clones were sequenced.

For screening on an EGFR antigen preparation, an original plasmid mixture comprising a 1:1 molar ratio of pT7340A : pT7591A gave rise to a final pUC18 library consisting of 84% pT7340A sequence and 16% pT7591A. With a 1:1 molar ratio of pT7340A : pT7340B or a 1:1 molar ratio of pT7340B : pT7591B, the final pUC18 library comprised 100% of the pT7340B sequence in both cases.

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